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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

ATTY.'S DOCKET: FUCHS=2A

In re Application of: ) Art Unit: 1647  
FUCHS et al. )  
Appln. No.: 09/820,339 ) Examiner: R. C. Hayes  
Date Filed: March 29, 2001 ) Washington, D.C.  
For: RECOMBINANT FRAGMENTS OF ) Confirmation No. 3100  
THE HUMAN ACETYLCHOLINE )  
RECEPTOR AND THEIR... ) May 27, 2004

REQUEST FOR PRIORITY

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Sir:

In accordance with the provisions of 37 CFR §1.55 and the requirements of 35 U.S.C. §119, filed herewith a certified copy of:

Appln. No.: 120792	Filed: May 7, 1997
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It is respectfully requested that applicant be granted the benefit of the priority date of the foreign application.

Respectfully submitted,

BROWDY AND NEIMARK, P.L.L.C.  
Attorneys for Applicant(s)

By

  
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משרד המשפטים  
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היום

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Certified

מספר : <b>120792</b> Number
תאריך : <b>07-05-1997</b> Date
הוקדס/נדחת Ante/Post-dated

בקשה לפטנט  
Application for Patent

אני (שם המבקש, מענו ולגבי גוף מאוגד - מקום התאגדותו)  
(Name and address of applicant, and in case of body corporate-place of incorporation)

ידע חברה למחקר ופיתוח בע"מ, חברת ישראלית, ליד מכון ויצמן למדע, ת.ד. 95, רחובות 76100  
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Inventors: Sara Fuchs, Miriam C. Souroujon, Dora Barchan

בעל אמצעה מכח ההעברה  
Assignment  
of an invention the title of which is  
Owner, by virtue of

פוליפפטידים, מולקולות DNA המקודדות להם ותכשורי רוקחות  
המכילים את הפוליפפטידים לטיפול ואיבחו של מייאסטניה גרavis  
(בעברית)  
(Hebrew)

Polypeptides, DNA molecules coding therefor and pharmaceutical compositions  
comprising the polypeptides for treatment and diagnosis of myasthenia gravis  
(באנגלית)  
(English)

מבקש בזאת כי ינתן לי עליה פטנט  
hereby apply for a patent to be granted to me in respect thereof.

*בקשת חלוקה - Application of Division		*בקשת פטנט נוסף - Application for Patent Addition		דרישה דין קדימה Priority Claim		
מספר/סימן Number/Mark	תאריך Date	מדינה האנוגד Convention Country				
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* פוי כח: כללית / מיוחד - רצוף בהז / עד יוגש P.O.A.: general / individual-attached / to be filed later - filed in..... 110696 הוגש בעניין..... case						
המוע למסירת מסמכים בישראל 9702 Address for Service in Israel						
חתימת המבקש Signature of Applicant For the Applicants, <b>Paulina Ben-Ami</b> Paulina Ben-Ami Patent Attorney			6 mai שנת..... 1997 of the year May This			
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**Polypeptides, DNA molecules coding therefor and pharmaceutical compositions comprising the polypeptides for treatment and diagnosis of myasthenia gravis**

**פוליפפטידים, מולקולות DNA המקודדות להם  
ותכשיiri רוקחות המפעילים את הפוליפפטידים לטיפול ואיבחון  
של מיאסטניה גראVIS**

**Yeda Research and Development Co. Ltd.**

Inventors: Sara Fuchs, Miriam C. Souroujon, Dora Barchan

**ידע חברה למחקר ופיתוח בע"מ**

ממצאים: שרה פוקס, מרים سورויגון, דורה ברמן

## FIELD OF THE INVENTION

The present invention relates to polypeptides capable of modulating the autoimmune response to acetylcholine receptor, and more particularly to polypeptides corresponding entirely or partially to the extracellular domain of human acetylcholine receptor (hAChR)  $\alpha$ -subunit, which polypeptides are useful in the diagnosis and treatment of myasthenia gravis (MG), and to DNA molecules encoding said polypeptides.

## BACKGROUND OF THE INVENTION

Myasthenia gravis (MG) is a human autoimmune disorder characterized by muscle weakness and fatigability. In this disease, antibodies against the acetylcholine receptor (AChR) bind to the receptor and interfere with the transmission of signals from nerve to muscle at the neuromuscular junction [Patrick and Lindstrom, 1973].

The acetylcholine receptor molecule is a transmembrane glycoprotein consisting of five homologous subunits, organized in a barrel-staves-like structure around a central cation channel, in the stoichiometry of either  $\alpha_2\beta\gamma\delta$  in fetal, or  $\alpha_2\beta\epsilon\delta$  in mature, muscle [Karlin, 1980; Changeux et al., 1984]. Noda et al. (1983) described the cloning and sequence analysis of human genomic DNA encoding the  $\alpha$ -subunit precursor of muscle acetylcholine receptor, and Schoepfer et al. (1988) reported the cloning of the  $\alpha$ -subunit cDNA from the human cell line TE671. Human muscle AChR  $\alpha$ -subunit exists in two forms, one of which has 25 additional amino acid residues inserted between positions 58 and 59, that are coded by the 75bp exon p3A [Beeson et al., 1990]. The  $\alpha$ -subunit of AChR contains both the site for acetylcholine binding and the main targets for anti-AChR antibodies.

The autoimmune response in myasthenia gravis is directed mainly towards the extracellular domain of the AChR  $\alpha$ -subunit (amino acids 1-210), and within it, primarily towards the main immunogenic region (MIR) encompassing amino acids 61-76 [Tzartos and Lindstrom, 1980; Tzartos et al., 1987; Loutrari et al., 1992].

The involvement of antibodies directed to the MIR and to the ligand binding site of AChR in the autoimmune process can be assessed by the ability of monoclonal antibodies (mAbs) with these specificities to passively transfer experimental autoimmune myasthenia gravis

(EAMG) into animals. Examples of such antibodies are mAb 198, mAb 195, mAb 202 and mAb 35 directed towards the MIR of the extracellular portion of hAChR  $\alpha$ -subunit [Sophianos and Tzartos, 1989], and mAb 5.5 directed towards the binding site of AChR [Mochly-Rosen and Fuchs, 1981]. The anti-MIR antibodies exert their effect by crosslinking AChRs on the muscle surface thereby accelerating their degradation, and the anti-binding site mAbs by blocking and competing with acetylcholine [Souroujon et al., 1986; Asher et al., 1993; Loutrari et al., 1992a]. Anti-MIR mAbs have also been shown to accelerate the degradation of AChR in the human cell line TE671 [Loutrari et al., 1992].

MG is currently treated by acetylcholinesterase inhibitors and by non-specific immunosuppressive drugs that have deleterious side effects. It would be preferable to treat MG with a method that involves antigen-specific immunotherapy but leaves the overall immune response intact. One such strategy of specific therapy could involve the administration of derivatives of AChR that do not induce myasthenia but are capable of affecting the immunopathogenic antibodies. However, since the anti-AChR antibody repertoire in myasthenia gravis has been shown to be polyclonal and heterogeneous [Drachman, 1994], the regulation of the disease requires modulation of many antibody specificities.

Previous studies at the laboratory of the present inventors were directed towards modulating the anti-AChR response and EAMG by either denatured derivatives of Torpedo AChR, e.g. the reduced and carboxymethylated derivative, RCM-AChR [Bartfeld and Fuchs, 1978], synthetic peptides corresponding to specific regions of AChR [Souroujon et al., 1992; Souroujon et al., 1993], or mimotopes selected from an epitope library [Balass et al., 1993]. The Torpedo RCM-AChR did not induce EAMG in rabbits and was effective in suppressing the disease. However, RCM-AChR did induce EAMG in rats. The experiments carried out with the synthetic peptides and mimotopes were only partially successful in neutralizing MG autoimmune response, probably due to the incorrect folding of the short peptides that were recognized by only a portion of the anti-AChR antibodies.

MG is currently diagnosed by testing for antibodies against AChR by radioimmunoassay wherein the antigen is crude AChR extracted from human muscle or TE671 cells. This test presents some drawbacks, namely the antigen is not readily available and, in addition, the antibody titers detected are not well correlated with disease severity.

Thus, both a safe and effective treatment for MG, as well as a reliable and convenient diagnosis test, are much in desire.

## SUMMARY OF THE INVENTION

It has now been found according to the present invention that polypeptides comprising sequences corresponding to the entire extracellular domain of the human AChR  $\alpha$ -subunit, or to fragments thereof, are capable of modulating the autoimmune response to AChR. Said polypeptides, herein referred to as "biologically active" polypeptides, affect the antigenic modulation of AChR in TE671 cells in vitro, and modulate the course of EAMG in vivo. The biologically active polypeptides were effective in suppressing the disease both in EAMG that was passively transferred by monoclonal anti-AChR antibodies, and in EAMG that was actively induced by immunization with AChR, while they did not induce any symptoms of MG in the rat model system.

Thus, the present invention provides, in one aspect, a polypeptide capable of modulating the autoimmune response of an individual to acetylcholine receptor, said polypeptide being selected from the group consisting of:

- (i) a polypeptide corresponding to amino acid residues 1-210 of the human acetylcholine receptor (hAChR)  $\alpha$ -subunit sequence depicted in Fig.1 ( $H\alpha 1$ -210), in which is inserted, between amino acid residues 58 and 59, a sequence of 25 amino acid residues encoded by the p3A exon of the hAChR  $\alpha$ -subunit gene, depicted in Fig.2 ( $H\alpha 1$ -210+p3A);
- (ii) a polypeptide corresponding to amino acid residues 1-121 of the hAChR  $\alpha$ -subunit sequence depicted in Fig.1 ( $H\alpha 1$ -121);
- (iii) a polypeptide corresponding to amino acid residues 1-121 of the hAChR  $\alpha$ -subunit sequence depicted in Fig.1, in which is inserted, between amino acid residues 58 and 59, a sequence of 25 amino acid residues encoded by the p3A exon of the hAChR  $\alpha$  subunit gene, depicted in Fig.2 ( $H\alpha 1$ -121+p3A);
- (iv) a polypeptide corresponding to amino acid residues 122-210 of the hAChR  $\alpha$ -subunit sequence depicted in Fig.1 ( $H\alpha 1$ 22-210);
- (v) a polypeptide as in (i) to (iv) or the polypeptide  $H\alpha 1$ -210 in which one or more amino acid residues have been added, deleted or substituted by other amino acid residues in a manner that the resulting polypeptide is capable of modulating the autoimmune response to acetylcholine receptor;
- (vi) a fragment of a polypeptide as in (i) to (v), which fragment is capable of modulating the autoimmune response to acetylcholine receptor;

(vii) a polypeptide comprising two or more fragments as in (vi) fused together with or without a spacer;

(viii) a polypeptide or a fragment as defined in (i)-(vii) or the polypeptide H $\alpha$ 1-210 fused to an additional polypeptide at its N- and/or C-terminal; and

(ix) soluble forms, chemical derivatives and salts of a polypeptide or a fragment as defined in (i)-(viii).

Preferred polypeptides according to the invention are H $\alpha$ 1-121 and H $\alpha$ 122-210, and in particular H $\alpha$ 1-210+p3A, H $\alpha$ 1-121+p3A, optionally fused to an additional polypeptide e.g. GST, and H $\alpha$ 1-210 similarly fused.

Preferably a fragment of H $\alpha$ 1-121 comprises at least the amino acid residues 61-76 of the hAChR  $\alpha$ -subunit sequence depicted in Fig.1, and a fragment of H $\alpha$ 122-210 comprises at least the amino acid residues 184-210 of the hAChR  $\alpha$ -subunit sequence depicted in Fig.1.

In another aspect, the invention encompasses a DNA molecule coding for a biologically active polypeptide according to the invention. Said DNA molecules may be selected from genomic DNA, cDNA or recombinant DNA or may be synthetically produced.

In particular the invention provides a DNA molecule comprising a nucleotide sequence coding for a polypeptide of the invention, said DNA molecule being selected from the group consisting of:

(i) a DNA molecule comprising the sequence of nucleotides 1 to 630, depicted in Fig.1, in which the sequence of the p3A exon of the hAChR  $\alpha$ -subunit gene, depicted in Fig.2, is inserted between nucleotides 174 and 175;

(ii) a DNA molecule comprising the sequence of nucleotides 1 to 363 depicted in Fig.1;

(iii) a DNA molecule comprising the sequence of nucleotides 1 to 363 depicted in Fig.1, in which the sequence of the p3A exon of the hAChR  $\alpha$ -subunit gene, depicted in Fig.2, is inserted between nucleotides 174 and 175;

(iv) a DNA molecule comprising the sequence of nucleotides 364 to 630 depicted in Fig.1;

(v) DNA molecules which are degenerate, as a result of the genetic code, to the DNA sequences of (i) to (iv) and which code for a polypeptide coded for by any one of the DNA sequences of (i) to (iv);

(vi) a DNA molecule having a coding nucleotide sequence which is at least 70% homologous to any one of the DNA sequences of (i) to (v) or to the DNA sequence coding for H $\alpha$ 1-210;

(vii) a DNA molecule as in (i) to (iv) or the DNA molecule coding for H $\alpha$ 1-210 in which one or more codons has been added, replaced or deleted in a manner that the polypeptide coded for by said sequence is capable of modulating the autoimmune response to acetylcholine receptor;

(viii) a fragment of a DNA molecule as in (i)-(vii) which codes for a polypeptide capable of modulating the autoimmune response to acetylcholine receptor;

(ix) a DNA molecule comprising two or more fragments of (viii) fused together with or without a spacer, and which codes for a polypeptide capable of modulating the autoimmune response to acetylcholine receptor; and

(x) a DNA molecule comprising a nucleic acid sequence as defined in (i)-(ix) or the DNA sequence coding for H $\alpha$ 1-210 fused to additional coding DNA sequences at its 3' and/or 5' end.

Preferred DNA molecules according to the invention are those comprising the sequence of nucleotides 1-363 and 364-630, depicted in Fig.1, coding for H $\alpha$ 1-121 and H $\alpha$ 122-210, respectively, and particularly the sequences of nucleotides 1-630 and 1-363, depicted in Fig.1, in which the sequence of the p3A exon of hAChR  $\alpha$ -subunit gene, depicted in Fig.2, is inserted between nucleotides 174 and 175, said DNA molecules coding, respectively, for H $\alpha$ 1-210+p3A and H $\alpha$ 1-121+p3A that comprise the additional 25 amino acid residues coded for by the p3A exon of the hAChR  $\alpha$ -subunit gene, as well as a DNA molecule coding for H $\alpha$ 1-210 fused to additional coding DNA sequences e.g. the sequence coding for GST.

Preferably, a fragment DNA molecule according to the invention codes for a polypeptide comprising at least the amino acid residues 61-76 and/or 184-210 of the hAChR  $\alpha$ -subunit sequence depicted in Fig.1.

In still other aspects, the invention provides replicable expression vehicles comprising a DNA molecule of the invention and prokaryotic or eukaryotic host cells transformed therewith.

A further aspect of the invention relates to a process for preparation of the polypeptides of the invention comprising culturing, under conditions promoting expression, host cells transformed by replicable expression vehicles comprising the DNA molecules of the invention, and isolating the expressed polypeptides.

In yet another aspect, the present invention provides pharmaceutical compositions comprising a pharmaceutically acceptable carrier and, as active ingredient, a polypeptide selected from the group consisting of the polypeptides of the invention and a polypeptide comprising the amino acid residues 1-210 of the hAChR  $\alpha$ -subunit depicted in Fig. 1 ( $\text{H}\alpha 1$ -210), soluble forms, salts and chemical derivatives thereof. The polypeptide  $\text{H}\alpha 1$ -210 was previously described in the literature as a polypeptide which induces myasthenia gravis [Lennon et al., 1991], but the use of this polypeptide for alleviation and/or treatment of myasthenia gravis is herein disclosed for the first time.

In still another aspect, the present invention provides methods for diagnosis and for alleviation and/or treatment of myasthenia gravis using the polypeptides and pharmaceutical compositions of the invention.

#### **BRIEF DESCRIPTION OF THE DRAWINGS**

**Fig. 1** depicts the nucleotide sequence (upper line) and the amino acid sequence coded thereby (lower line) corresponding to the extracellular domain of the hAChR  $\alpha$ -subunit.

**Fig. 2** depicts the nucleotide sequence (upper line) and amino acid sequence coded thereby (lower line) corresponding to the p3A exon of the hAChR  $\alpha$ -subunit gene.

**Figs. 3A-C** depict Coomassie staining (3A) and Western blots with mAb 198 (3B) or mAb 5.5 (3C) of  $\text{H}\alpha 1$ -210+p3A,  $\text{H}\alpha 1$ -210,  $\text{H}\alpha 1$ -121+3pA,  $\text{H}\alpha 1$ -121 and  $\text{H}\alpha 122$ -210 fused to glutathione S-transferase (GST) at the N-terminal (lanes 1 to 5, respectively). GST alone (lane 6) served as a control.

**Fig. 4** depicts results of an ELISA assay showing binding of mAb198 to  $\text{H}\alpha 1$ -210+p3A (filled squares),  $\text{H}\alpha 1$ -210 (open squares),  $\text{H}\alpha 1$ -121+p3A (filled circles) and  $\text{H}\alpha 1$ -121 (open circles).

**Fig. 5** depicts results of an ELISA assay showing binding to  $\text{H}\alpha 1$ -210+3pA of mAb 198 (filled squares), mAb 5.5 (open triangles), mAb 195 (filled "upside down" triangles), mAb 202 (filled "upright" triangles) and mAb 35 (open circles).

**Fig. 6** depicts results of an ELISA assay demonstrating inhibition of mAb198 (0.1 $\mu$ g/well) binding to AChR by the following polypeptides:  $\text{H}\alpha 1$ -210+3pA (filled squares),  $\text{H}\alpha 1$ -210 (open squares),  $\text{H}\alpha 1$ -121+3pA (filled circles),  $\text{H}\alpha 1$ -121 (open circles) and GST (filled triangles), at concentrations of 0.05-10  $\mu$ g/well.

**Fig. 7** depicts the inhibition effect of the polypeptides of the invention on AChR degradation induced by mAb 198. TE671 cells were incubated with (a) medium, (b) 1 µg/ml mAb 198, (c-g) 1 µg/ml of mAb 198 preincubated with either H $\alpha$ 1-121 (hatched columns) or with H $\alpha$ 122-210 (dark columns) at concentrations of 10 (c), 25 (d), 50 (e), 100 (f) and 200 (g) µg/ml. Residual AChR was monitored by measuring  $\alpha$ -bungarotoxin ( $\alpha$ -BTX) binding sites.

**Fig. 8** depicts the effect of H $\alpha$ 1-121+p3A on AChR degradation induced by different mAbs. Residual AChR was monitored by measuring  $\alpha$ -BTX binding sites. TE671 cells were incubated with medium alone (leftmost column) or with added mAb 198 (1 µg/ml), mAb 35 (1 µg/ml), mAb 195 (5 µg/ml) or mAb 202 (5 µg/ml) either without (dotted columns) or following preincubation of the mAbs with H $\alpha$ 1-121+p3A (hatched columns).

**Fig. 9** depicts the effect of intranasal administration to rats of H $\alpha$ 1-210+p3A, H $\alpha$ 1-121+p3A or H $\alpha$ 122-210 (each fused to GST at the N-terminal) on the in vitro lymphocyte proliferative response to AChR. Rats were pretreated with either GST (control), H $\alpha$ 122-210, H $\alpha$ 1-121+p3A or H $\alpha$ 1-210+p3A, and were immunized 3 days later with Torpedo AChR. Three weeks after immunization, the rats were sacrificed and their lymph nodes were restimulated in vitro by 5.0, 2.5 and 0.25 µg/ $\mu$ l AChR. Results are presented as [ $^3$ H]-thymidine cpm incorporated into cells after subtraction of cpm in the presence of medium alone.

#### **DETAILED DESCRIPTION OF THE INVENTION**

Patients with the neuromuscular disease myasthenia gravis are characterized by the pathogenic autoantibodies they develop directed towards acetylcholine receptor (AChR) [Aharonov et al., 1975]. The  $\alpha$ -subunit of AChR appears to be the prime target for these pathogenic autoantibodies, and within it especially the extracellular domain.

Human muscle AChR  $\alpha$ -subunit exists as two isoforms consisting of 437 and 462 amino acid residues [Beeson et al., 1990]. The two isoforms are identical in their amino acid composition except for a sequence of 25 additional amino acid residues inserted after position 58 in the extracellular domain of the longer variant. These additional amino acids are encoded by the 75bp exon p3A.

It was found according to the present invention that the polypeptides herein designated H $\alpha$ 1-210, H $\alpha$ 1-210+p3A, H $\alpha$ 1-121, H $\alpha$ 1-121+p3A and H $\alpha$ 122-210 are capable of modulating the autoimmune response to acetylcholine receptor (AChR) and of suppressing experimental myasthenia gravis in animal models.

The present invention thus relates to the novel polypeptides H $\alpha$ 1-121, H $\alpha$ 1-121+p3A, H $\alpha$ 122-210 and H $\alpha$ 1-210+p3A as well as to analogs, fragments, fused derivatives, chemical derivatives and salts thereof, and to novel analogs, fragments, fused derivatives, chemical derivatives and salts of the peptide H $\alpha$ 1-210.

Analogs according to the invention are polypeptides in which one or more amino acid residues have been added to, replaced in or deleted from the original polypeptide in a manner that the resulting polypeptide retains its biological activity. These analogs may be prepared by known synthesis procedures and/or by genetic engineering methods, for example by expressing a DNA molecule modified by site-directed mutagenesis.

Biologically active fragments of the polypeptides encompassed by the present invention include preferably polypeptides comprising at least the amino acid residues 61 to 76 and/or 184 to 210 of the hAChR  $\alpha$ -subunit sequence, representing, respectively, the main immunogenic region (MIR) and the acetylcholine binding site of the hAChR  $\alpha$ -subunit. A fragment comprising at least amino acid residues 61 to 76 is a preferred fragment according to the invention. Also included in the invention are polypeptides comprising two or more fragments as mentioned above which are fused together with or without a spacer.

Chemical derivatives of polypeptides of the invention include modifications of functional groups at side chains of the amino acid residues, or at the N- and/or C-terminal groups. Examples of such derivatives include, but are not limited to, esters of carboxyl and hydroxy groups, amides of carboxyl groups generated by reaction with ammonia or with primary or secondary amines and N-acyl derivatives of free amino groups. Cyclic forms of the polypeptides containing a disulfide bridge between two cysteines residues to stabilize the molecule are also encompassed by the invention.

The salts of the polypeptides of the invention are pharmaceutically acceptable, i.e. they do not destroy the biological activity of the polypeptide, do not confer toxic properties on compositions containing them and do not induce adverse effects. The term "salts" refers to salts of carboxyl groups as well as to acid addition salts of amino groups of the polypeptide molecule.

A polypeptide of the invention, or a fragment thereof, may be fused to an additional polypeptide at its N- and/or C-terminal. For example, recombinant polypeptides were prepared where H $\alpha$ 1-210, H $\alpha$ 1-210+p3A, H $\alpha$ 1-121, H $\alpha$ 1-121+p3A or H $\alpha$ 122-210 were fused to glutathione S-transferase (GST) at the N-terminal, and these molecules were capable of modulating the immune response to AChR. Other polypeptides may be fused to the N- and/or C-

terminal of a polypeptide of the invention provided that the fusion does not significantly impair the three dimensional structure of the resulting polypeptide in a way that prevents it from being recognized by anti-AChR antibodies.

A polypeptide according to the invention corresponding entirely or partially to the extracellular domain of the hAChR  $\alpha$ -subunit should be capable of affecting the immunopathogenic response without inducing myasthenia gravis by itself. In order to neutralize the anti-AChR antibodies, the polypeptide should be at least partially correctly folded, so that it will be recognized by said antibodies. Furthermore, since the anti-AChR antibody repertoire in myasthenia gravis has been shown to be polyclonal and heterogeneous [Drachman, 1994], the regulation of myasthenia gravis requires modulation of many antibody specificities. The recombinant polypeptides according to the invention have, indeed, shown to have a broad specificity as demonstrated by their ability to protect AChR in TE671 cells against antigenic modulation induced by a series of anti-AChR mAbs (Fig 8) or by polyclonal anti-AChR antibodies from myasthenic rats (data not shown).

The three-dimensional arrangement of a polypeptide of the invention seems to be of crucial importance for its biological activity. It was shown in several experiments (see Fig. 3b, Fig. 3c, Fig. 4 and Fig. 6) that the polypeptides comprising the additional 25 amino acid residues coded for by exon p3A, namely H $\alpha$ 1-121+p3A and H $\alpha$ 1-210+p3A, bind to anti-AChR antibodies better than the shorter variants H $\alpha$ 1-121 and H $\alpha$ 1-210, and are also more potent in their protection effect in TE671 cells in vitro and in EAMG in vivo. Thus H $\alpha$ 1-121+p3A and H $\alpha$ 1-210+p3A are the most preferred polypeptides according to the invention.

The major binding sites of the anti-AChR mAbs inhibited by the polypeptides of the invention do not seem to reside within the stretch of amino acids encoded by p3A. Therefore, it seems likely that the different specificity of these mAbs towards the polypeptides with and without the p3A encoded sequence reflects conformational changes due to the presence of said extra encoded sequence. Conformational differences could also explain why mAb198 binds well to the polypeptides containing the p3A encoded sequence, but was unable to immunoprecipitate an oocyte-expressed  $\alpha$ -subunit containing this sequence [Newland et al., 1995].

A polypeptide of the invention may be produced by means of recombinant technology or synthetically employing methods well known in the art.

Recombinant polypeptides according to the invention are prepared by culturing host cells transformed by a suitable expression vector containing a DNA molecule of the invention under

conditions promoting expression, and isolating the expressed polypeptide, using standard techniques well known in the art (see, for example, Sambrook et al., 1989; Ausubel et al., 1993).

Soluble forms of the polypeptides that constitute a preferred embodiment of the invention may be generated by suitable chemical modification of natural amino acid residues in the polypeptide, or by substitution of said natural amino acid residues by suitable hydrophilic natural or non-natural amino acids. Alternatively, solubility may be induced by fusion of a polypeptide of the invention to a highly soluble polypeptide partner, such as glutathione S-transferase (GST), immunoglobulin or a fragment thereof, maltose binding protein (MBP), thioredoxin or Influenza non-structural protein 1 (NS1). The additional polypeptide may be fused at the N- or C-terminal of the polypeptide of the invention, and it should not significantly impair the three dimensional structure of the polypeptide corresponding to the hAChR  $\alpha$ -subunit domain.

The fused polypeptide of the invention may be used as such, or it may be subjected to further processing in which an active polypeptide of the invention is released. Insertion of a target sequence that is cleavable by specific proteases, such as V8 protease, enterokinase, thrombin or factor Xa, enables the release of the original polypeptide from the recombinant expressed fused polypeptide.

A DNA molecule according to the invention comprises a nucleotide sequence coding for a biologically active polypeptide of the invention. The DNA molecule may be from any origin including non-human sources, and may be selected from genomic DNA, cDNA, recombinant DNA, PCR-produced or synthetically produced DNA.

Preferred DNA molecules are those comprising the sequence of nucleotides 1-363 and 364-630 of the hAChR  $\alpha$ -subunit (depicted in Fig.1) coding for H $\alpha$ 1-121 and H $\alpha$ 122-210, respectively, and particularly the sequences of nucleotides 1-630 and 1-363 of the hAChR  $\alpha$ -subunit in which the sequence of the p3A exon of the hAChR  $\alpha$ -subunit gene (depicted in Fig.2) is inserted between nucleotides 174 and 175, hence coding, respectively, for H $\alpha$ 1-210+p3A and H $\alpha$ 1-121+p3A.

A fused DNA molecule according to the invention comprises a nucleic acid sequence coding for a polypeptide of the invention in fusion to additional coding DNA sequences at its 3' and/or 5' end. The added DNA sequence may code for a polypeptide endowing the expressed fused polypeptide with favourable characteristics for its purification or for performing its

biological activity, i.e. conferring on the original polypeptide molecule a preferred configuration or high solubility.

A DNA molecule of the invention may be directly isolated from human genomic DNA or cDNA by standard means known in the art involving subcloning genomic or cDNA fractions into a replicable vector, amplifying the subcloned fragments, detecting the relevant clones by their hybridization to the DNA molecules of the invention or fragments thereof, followed by their isolation, for example as described in Sambrook et al., eds. "Molecular Cloning: A Laboratory Manual", 2nd ed., Cold Spring Harbor Press, 1989; and in "Current Protocols in Molecular Biology" Current Protocols, Ausubel et al., eds., 1993.

DNA molecules which are at least 70% homologous to H $\alpha$ 1-210, H $\alpha$ 1-210+p3A, H $\alpha$ 1-121, H $\alpha$ 1-121+p3A or H $\alpha$ 122-210 may be isolated by subjecting a population of cloned genomic DNA or cDNA molecules to hybridization with the above synthesized DNA molecules or fragments thereof under stringent conditions, and isolating the hybridized clones. The term "stringent conditions" refers to hybridization and subsequent washing conditions conventionally referred to in the art as "stringent" (see Sambrook et al. and Ausubel et al., supra).

Alternatively, a DNA molecule of the invention may be PCR-produced as described for example in Example 1 hereinafter. In general, the PCR-production procedure comprises total RNA purification from relevant cells and generation of first strand cDNA by reverse transcriptase, using either an antisense oligonucleotide mixture or oligo (dT) as a primer. A cDNA fragment may be then amplified in a polymerase chain reaction (PCR) using appropriate sense and antisense primers flanking the target cDNA fragment. The PCR primers may include restriction sites to be used for restriction enzyme digestion followed by cloning into a suitable vector.

Cloning of a DNA molecule of the invention within an appropriate expression vehicle and expression in a suitable host cell enables production and isolation of a biologically active polypeptide or fragment thereof. For this purpose, the DNA molecule is incorporated into a plasmid or viral vector preferably capable of autonomous replication in a recipient host cell of choice. Optionally, the DNA molecule may be cloned into an expression vector in frame with additional coding sequences at its 5' and/or 3' end, e.g. the pGEX plasmid vectors that contain GST coding sequences fused upstream to the cloning site. The recombinant expression vector is then used to transform an appropriate prokaryotic or eukaryotic host cell that, under inducing conditions, expresses the polypeptide itself or fused to an additional sequence. In the latter case,

insertion of a recognition site for a protease, enables at will the release of the cloned polypeptide from the additional fused polypeptide.

Vectors used in prokaryotic cells include, but are not limited to, plasmids capable of replication in E. Coli, (for example, pGEX), and bacteriophage vectors such as λgt11, λgt18-23 M13 derived vectors etc.

Vectors for use in eukaryotic cells include, but are not limited to, viruses such as retroviruses and vaccinia.

A vector construct containing the DNA molecule of the invention is then introduced into an appropriate host cell by any of a variety of suitable means known in the art, such as transformation, transfection, lipofection, conjugation, protoplast fusion, electroporation, calcium phosphate precipitation, direct microinjection, etc.

Suitable host cells useful in the invention are prokaryotic cells which include, but are not limited to E. Coli, and more preferably, eukaryotic hosts which include, but are not limited to, yeast cells such as *Saccharomyces cerevisiae*, or insect cell lines, for example *Spodoptera frugiperda* (*Sf9*) cells which are commonly used with the baculovirus expression system, or mammalian cells such as Chinese hamster ovary (CHO) cell lines.

Eukaryotic cells are the preferred hosts in expression systems for producing the polypeptides of the invention since they can perform the correct post-translational processing to confer the right conformation on said polypeptides. However, since partially correctly folded polypeptides may also be biologically active, prokaryotic expression systems may also be useful, especially for the production of large amount of polypeptides.

In another aspect, the present invention relates to pharmaceutical compositions comprising a pharmaceutically acceptable carrier and, as active ingredient, a polypeptide selected from a polypeptide of the invention and a polypeptide comprising the amino acid residues 1-210 of hAChR α-subunit depicted in Fig.1, soluble forms, salts and chemical derivatives thereof.

The pharmaceutical compositions are for use in the alleviation and/or treatment of myasthenia gravis. The pharmaceutical compositions of the invention may be in any suitable form for administration of polypeptides known in the art, e.g. by injection, inhalation, orally, nasally etc.

Appropriate pharmaceutically acceptable carriers include physiological carriers, such as water and oils and excipients such as stabilizers and preservative agents. Saline solutions and aqueous dextrose and glycerol solution are suitable for injectable solutions. The active

ingredient may also be prepared as a lyophilized dry compound, possibly as a salt, or as a conjugate with a solid carrier/support such as dextran, natural and modified celluloses, etc. The pharmaceutically acceptable carrier of choice will be determined depending on the route the pharmaceutical composition will be administered.

The dosage of the polypeptide and the schedule of the treatment should depend on the route of administration, the patient condition, age and genetic background and will be determined by a skilled professional person. For example, based on animal studies, it was found that dosage ranges of about 1.4 µg - 14 mg and 0.14µg - 0.7 mg/ Kg human body weight are suitable for oral and nasal administration, respectively, in humans.

The invention further provides a method for alleviation or treatment of myasthenia gravis which comprises administering to an individual in need thereof an effective amount of a polypeptide selected from a polypeptide of the invention and the polypeptide H $\alpha$ 1-210, a soluble form, a chemical derivative or a salt thereof.

In contrast to the current methods of treatment of MG using non-specific immunosuppressive drugs, such as steroids, azathioprine or cyclosporine, the method of present invention is directed to an antigen-specific immunotherapy strategy thus suppressing only the adverse autoimmune responses while leaving the overall immune system of the patient intact.

Preferred routes of administration of the polypeptides according to the invention are the nasal and oral routes.

Nasal tolerization has several advantages as a treatment modality in comparison with oral tolerization: it requires smaller doses of antigen, is more convenient to use and does not require soybean trypsin inhibitor (STI) used in oral tolerance to inhibit the degradation of the antigen in the gastrointestinal tract. Some successful attempts to modulate experimental autoimmune diseases in animal models by nasal administration of the autoantigen have been recently reported. Thus, Weiner et al. [1994] showed that inhalation of aerosols containing myelin basic protein (MBP) abrogated the clinical symptoms of EAE and significantly reduced the CNS inflammation, DTH reaction and antibody titer to MBP; Dick et al. [1993] reported that nasal administration of retinal extract inhibited the induction of experimental allergic uveitis (EAU) by immunization with this extract; and Ma et al. [1995] demonstrated that nasal administration of the antigen Torpedo AChR diminished the incidence and severity of clinical muscle weakness characteristic of EAMG following immunization with said antigen.

The pharmaceutical compositions of the present invention are also useful for diagnosis of myasthenia gravis whereby anti-AChR antibodies in the serum of a patient are determined by employing one or more polypeptides of the invention as the test antigen and bound anti-AChR antibody titers indicate the presence of myasthenia gravis.

For the diagnostic test, a serum aliquote of a patient is brought in contact with one or more polypeptides, incubated for about 1 hr to overnight at 4°-37°C, followed by the determination of the amount of anti-AChR antibodies bound to the polypeptides by quantitative detection assays known in the art.

In one embodiment, the diagnostic test is carried out with immobilized polypeptides in an assay comprising the following steps:

(i) immobilization of one or more polypeptides corresponding entirely or partially to the extracellular domain of human acetylcholine receptor on a suitable solid support;

(ii) incubation of said immobilized polypeptides of (i) with a serum sample from a patient for 1 hr to overnight at 4°-37°C; and

(iii) determination of the amount of the anti-AChR antibodies bound to the immobilized polypeptides fragments,

whereby detection of anti-AChR titers indicates the presence of myasthenia gravis.

The detection of the anti-AChR antibodies may be carried out with labeled anti-human antibodies or labeled Staphilococcus protein A. The label may be a radioactive or fluorescent tag, an enzyme conjugate or another biological recognition tag. Examples of radioactive tags are radioactive isotopes such as  $^{125}\text{I}$ ,  $^{35}\text{S}$ ,  $^{32}\text{P}$ ,  $^3\text{H}$ ,  $^{14}\text{C}$  etc, which are detected by a scintillation or a  $\gamma$ -counter or by autoradiography. Fluorescent tags are derived from fluorescent compounds such as fluorescein, isothiocyanate, rhodamine, phycoerythrin, phycocyanin, allophycocyanin, o-phthaldehyde and fluorescamine, and are detected by exposure of the bound fluorescent labeled antibody to light of the proper wavelength and monitoring the fluorescence.

Enzyme conjugates useful for detection purposes include, but are not limited to, maleate dehydrogenase, yeast alcohol dehydrogenase, horseradish peroxidase, alkaline phosphatase, beta-galactosidase, catalase and glucose-6-phosphate dehydrogenase. These enzymes are conjugated to the antibody or to protein A and can be monitored by the product they produce when exposed to the appropriate substrate. The chemical moiety thus released can be detected, for example, by chemiluminescence reaction or by spectrophotometry, fluorometry or visual means.

Diagnostic methods based on recognition of biological tags include, for example, coupling of protein A or of the anti-human antibodies to biotin. The biotinylated molecules then can be detected by avidin or streptavidin coupled to a fluorescent compound, to an enzyme such as peroxidase or to a radioactive isotope and the like.

In another embodiment, the diagnostic test is carried out with one or more soluble polypeptides pre-labeled by one of the foregoing labels and tags, whereby anti-AChR antibodies of the patient's serum bound to the polypeptides are separated from the free antigen by precipitation of the antigen-antibody complex by Staphilococcus protein A or anti-human antibodies, and anti-AChR titers are determined as described above.

The diagnostic assays according to the invention have the advantage of avoiding the need to extract the antigen from human tissues or cells, and also provides a more reproducible and safe way for MG detection. The use as antigens of polypeptides that recognize sub-populations of MG-related antibodies further provides a better means for correlating anti-AChR titers with disease severity.

The invention will now be illustrated by the following non-limiting examples and accompanying drawings.

## EXAMPLES

### MATERIALS AND METHODS

#### i) Monoclonal antibodies (mAb).

The following monoclonal antibodies were used: mAb directed towards the main immunogenic region (MIR) of the extracellular portion of hAChR  $\alpha$ -subunit [Sophianos and Tzartos, 1989]; mAb 198, mAb 195 and mAb 202 elicited in rats against human muscle AChR, and mAb 35 elicited in rats against electric eel AChR, but cross-reacted with AChR from other species, including human; and mAb 5.5 directed towards the binding site of AChR from other species, including human [Mochly-Rosen and Fuchs, 1981], elicited in mouse against Torpedo AChR.

#### ii) Antibody binding assays.

Binding of antibodies to AChR or to recombinant polypeptides corresponding entirely or partially to the extracellular domain of the hAChR  $\alpha$ -subunit was analyzed by ELISA. Wells of microtiter plates (Maxisorb, Nunc, Neptune, NJ) were coated by incubation overnight at 4°C

with either Torpedo AChR ( $1\mu\text{g}$  in  $100\ \mu\text{l}$  of PBS), or with one of the recombinant polypeptides of the invention ( $2\mu\text{g}$  in  $100\ \mu\text{l}$  of  $50\text{ mM}$  Tris buffer pH 8.0). Coated plates were washed three times with PBS containing 0.05% Tween-20, then wells were blocked by incubation for 1 hour at R.T. with 1% BSA and 1% hemoglobin in PBS, and the coated blocked plates were then washed and incubated overnight at  $4^\circ\text{C}$  with different amounts of antibody.

For inhibition experiments, each well was coated with  $1\ \mu\text{g}$  of Torpedo AChR and a polypeptide of the invention was preincubated with the mAb of choice for 30 min at R.T. before addition to the AChR-coated well. Following a washing step, bound mAb was determined by incubation for 1 hour at R.T. with 1:5000 dilution of alkaline phosphatase (AP)-conjugated goat anti-mouse IgGs (Jackson ImmunoResearch Labs, Inc., or Biomakor, Ness-Ziona, Israel). The bound antibody was detected by the enzymatic activity of AP using N-paranitrophenyl-phosphate as a substrate and determining by a microtiter plate reader at  $405\text{ nm}$ , the color developed after about 40 min.

iii) Determination of AChR content

AChR content was determined by measuring  $\alpha$ -bungarotoxin ( $\alpha$ -BTX) binding sites. Tested samples were derived from (a) muscle preparations or from (b) cells grown in a tissue culture.

a) For the muscle preparation, the procedure described by Souroujon et al. [1985] was essentially followed. Briefly, muscle tissue was removed and homogenized in a Sorvall omnimixer for 2 min. at full speed. Two volumes of Tris-HCl buffer, pH 7.5, containing  $0.1\text{ M}$  NaCl,  $1\text{ mM}$  EDTA,  $0.1\text{ mM}$  PMSF and  $0.5\text{ mM}$  NaN<sub>3</sub>, were used for homogenization. Homogenates were then centrifuged at  $48000\times g$  for 1 hr, washed once and recentrifuged as above. The homogenates were stirred overnight at  $4^\circ\text{C}$  in 2 volumes of the above Tris buffer containing Triton X-100 at a final concentration of 1%. The mixture was then centrifuged for 1 hr at  $100000\times g$  in a Beckman ultracentrifuge and the recovered supernatant was stored at  $70^\circ\text{C}$ . The AChR in the Triton extracts was determined by measuring the amount of  $^{125}\text{I}$ - $\alpha$ -BTX that coprecipitated with the receptor in ammonium sulfate at 35% saturation. Unbound toxin was removed by filtration through GF/C filters, and radioactivity retained on filters, i.e. toxin bound to receptor, was measured in a  $\gamma$  counter.

b) For determination of the AChR content in TE671 cells grown in tissue culture,  $^{125}\text{I}$ - $\alpha$ -BTX (final concentration about  $2\times 10^{-9}\text{M}$ ;  $10^6\text{ cpm}$ ) was added to a confluent cell culture in a

30 mm plate and incubated for an hour at 37°C. The cells were then washed four times with PBS, released with 1N NaOH and cell-bound radioactivity was evaluated in a  $\gamma$  counter, after deducting cpm in a control test tube containing an excess of unlabeled  $\alpha$ -BTX (final concentration  $10^{-6}$ M).

iv) Western blots

Electrophoresis of recombinant polypeptides corresponding to the entire or partial extracellular domain of the hAChR  $\alpha$ -subunit and their blotting were performed essentially as described [Wilson et al., 1985; Neumann et al., 1985]. The polypeptides were electrophoresed in 10% polyacrylamide gels and transferred to a nitrocellulose membrane. The membrane was preincubated in PBS containing 0.5% hemoglobin for 1h at R.T. before addition of 10 $\mu$ g/ml mAbs and incubation was carried out for additional 3 h at 37°C. The membranes were washed 4 times with PBS, once with PBS containing 0.5 % Triton X-100 and then incubated for 1h at 37°C with  $^{125}$ I-goat-anti-mouse Ig. After 5 washes, the blots were exposed to an X-ray sensitive film.

v) Antigenic modulation in TE671 cells

Antigenic modulation experiments were performed in 30mm 12-well plates using TE671 cell cultures. Cells ( $2 \times 10^4$ ) were plated in Dulbecco Modified Eagles medium (DMEM), containing 2mM L-glutamine, 10% fetal calf serum (FCS), and antibiotics (penicillin-streptomycin), and grown to confluence for 72 h. The antibodies were added in triplicates to culture wells at a concentration of 1 $\mu$ g/ml (and for mAbs 195 and 202 also at 5 $\mu$ g/ml) for 3 h. At the end of the incubation AChR content was determined by measuring  $^{125}$ I- $\alpha$ -BTX binding, as described in section (iii) above.

In order to test the effect of the polypeptides of the invention on the antigenic modulation induced by the antibodies, the mAbs were preincubated for 1h at 37°C with said polypeptides (at concentrations of 10-200 $\mu$ g/ml, as indicated), before their addition to the cell cultures, and the assay continued as described in section (ii) above.

vi) Passive transfer of EAMG to rats.

Lewis female rats (6 weeks old, approximate weight 120 gr) were used for passive transfer experiments, as previously described [Asher et al., 1993]. For the induction of EAMG, 80  $\mu$ g of the anti-MIR mAb 198 were injected i.p. to each rat. The tested polypeptide (1 mg) was preincubated with mAb 198 for 30 min at R.T., prior to the injection into rats. The rats were

observed for myasthenic symptoms and body weight. At 48 h after the administration of mAb, the animals were sacrificed and their leg muscles were removed for determination of the AChR content according to section (iii) above.

vii) Lymphocyte proliferation assay

Popliteal lymph nodes were aseptically removed and single cell suspensions were prepared in RPMI with 10 mM HEPES. An in vitro T-lymphocyte proliferative assay in response to AChR and the different polypeptides of the invention was performed as follows: Lymph node cells were suspended in RPMI at pH 7.4 containing 10 mM HEPES, supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids,  $5 \times 10^{-5}$ M  $\beta$ -mercaptoethanol and 0.5% normal rat serum, and plated in 96-well flat bottom plates (Corning;  $5 \times 10^5$  cells/well). Increasing concentrations of antigen (0.25 to 10  $\mu$ g/ml of AChR and 10 to 100  $\mu$ g/ml of a recombinant polypeptide of the invention), were then added to each well. Plates were incubated at 37°C, in 7.5% CO<sub>2</sub> and 90% humidity. Proliferation was assayed after 3 days by measuring incorporation of thymidine-methyl-[<sup>3</sup>H] into cells. Essentially, the cells were incubated with thymidine-methyl-[<sup>3</sup>H] (Rotem Ind. Ltd, Beer Sheva, Israel; 0.5 mCi/2.5ml) for 24 hours and then harvested and counted for radioactivity. Results are presented as incorporated cpm following subtraction of cpm in the presence of medium alone.

## EXAMPLES

### Example 1: Preparation of recombinant DNA molecules

DNA molecules encoding the biologically active polypeptides H $\alpha$ 1-210, H $\alpha$ 1-121, H $\alpha$ 122-210, H $\alpha$ 1-210+p3A and H $\alpha$ 1-121+p3A were synthesized as follows:

Total human RNA was prepared as described [Asher, 1988] from the TE671 cell line, which expresses the human muscle type nicotinic AChR [Schoepfer et al., 1988]. Preparation of cDNA and the polymerase chain reaction (PCR) were performed as described [Barchan et al., 1992]. The primers employed to amplify cDNA fragments corresponding to hAChR  $\alpha$ -subunit residues 1-210 (H $\alpha$ 1-210) and 1-210 containing the p3A exon (H $\alpha$ 1-210+p3A) [Beeson et al., 1990] were constructed with sites that enabled cloning into the fusion protein expression vector pGEX-2T. The primer at the 5' end, CCGGATCCGAACATGAGACC, corresponds to amino acid residues 1-5 of the human AChR  $\alpha$ -subunit (nucleotides coding for the first residue are bold), and had a BamHI site (underlined). The primer at the 3' end had an EcoRI site

(underlined) and was complementary to the DNA sequence coding for amino acid residues 206-210, **CGGAATTCCAGGCGCTGCATGAC**.

In a similar way the shorter clones H $\alpha$ 1-121, H $\alpha$ 1-121+p3A and H $\alpha$ 122-210 were derived by PCR using the above-mentioned H $\alpha$ 1-210 and H $\alpha$ 1-210+p3A clones as templates. For obtaining the two DNA molecules corresponding to amino acid residues 1-121 (with and without amino acid residues coded by the p3A exon), a primer complementary to the DNA sequence coding for amino acid residues 116-121 with an EcoRI site (underlined) **CGGAATTCTGGAGGTGTCCACGTGAT**, was used at the 3' end. For the 5' end, the primer described above corresponding to amino acid residues 1-5 was used. For cloning of the DNA coding for H $\alpha$ 122-210, the primer **CCGGATCCGCCATCTTAAAAGC** was used at the 5' end. This primer corresponds to amino acid residues 122-126 (nucleotides coding for residue 122 are in bold) and contains a BamHI site (underlined). The primer used at the 3' end was the same as described above for the DNA molecule coding for H $\alpha$ 1-210 (complementary to residues 206-210). The PCR amplified DNA sequences were subcloned into the BamHI-EcoRI sites of pGEX-2T expression vector (Pharmacia) [Smith and Johnson, 1988], in frame with glutathione S-transferase (GST) DNA sequences at the 5' end. All the cloned DNA molecules were sequenced in order to verify their sequence and then used to produce the recombinant polypeptides.

#### Example 2: Preparation of recombinant polypeptides

The different recombinant DNA molecules subcloned in pGEX-2T plasmid prepared in Example 1 were transfected into competent E. coli cells (strains JM101 or XL1-blue). The transformed bacteria were grown overnight in LB medium containing ampicillin, then diluted 1:150 in the medium and further grown for additional 3-5 hours. Induction of fused polypeptide expression was achieved by adding 0.5 mM IPTG (isopropyl  $\beta$ -D-thiogalactopyranoside) for 2 h. After expression, the bacterial suspension was centrifuged, cells were lysed by freezing and thawing the pellet and resuspended in phosphate-buffered saline (PBS, 10 ml). The preparation was sonicated for five 15-sec periods, and centrifuged for 15 min at 27000g. The expressed recombinant fused polypeptides were localized in the precipitate, probably in inclusion bodies. The fused polypeptides were solubilized in 1ml of 9M urea, the non-soluble fraction was removed by centrifugation for 45 min at 27000 x g, and the supernatant was diluted in 10ml of 50mM Tris buffer, pH 8.0 and dialyzed against the same buffer for 48 h with several changes.

After ultracentrifugation for 30 min at 100000 x g, the supernatant was divided into aliquots for storage at -80°C. The protein concentration, determined by the Lowry method, was 1-3 mg/ml, with a yield of 20-50 mg of total protein from one liter of bacterial suspension. The GST-fused polypeptides were isolated using a substrate affinity column according to Smith and Johnson, 1988. A Coomassie brilliant blue staining of the expressed GST-fused polypeptides run on 10% polyacrylamide gel is shown in Fig. 3A: from left to right, lanes 1-6, H $\alpha$ 1-210+p3A, H $\alpha$ 1-210, H $\alpha$ 1-121+p3A, H $\alpha$ 1-121, H $\alpha$ 122-210 and GST, appearing to have MW of 52.5, 50.0, 43.7, 41.2, 37.8 and 29.0 kD, respectively, in agreement with the expected MW calculated based on the encoded amino acid sequences of these polypeptides (see Fig. 1 and Fig. 2).

**Example 3: Immunochemical characterization of the recombinant polypeptides**

The recombinant polypeptides of Example 2 were further characterized by their binding to various anti-AChR mAbs as assayed by both Western blots (Fig. 3B- mAb 198; Fig. 3C- mAb 5.5) and by ELISA (Fig. 4 and Fig. 5).

The recombinant polypeptides (20 µg each) were electrophoresed, blotted onto nitrocellulose membrane, and incubated with different mAbs as described in Materials and Methods, section (iv). Fig. 3B shows that mAb 198, which is directed to the MIR, bound to the polypeptide corresponding to the entire extracellular portion of the hAChR  $\alpha$ -subunit (H $\alpha$ 1-210) and to its shorter derivative (H $\alpha$ 1-121), that contains the MIR, as well as to their variants including the additional p3A encoded sequence H $\alpha$ 1-210+p3A and H $\alpha$ 1-121+p3A. As expected, mAb 198 did not bind to H $\alpha$ 122-210, which does not include MIR, or to the GST protein itself.

mAb 5.5, which is directed to the binding site of AChR [Mochly-Rosen and Fuchs, 1981], bound to H $\alpha$ 1-210, H $\alpha$ 1-210+p3A and to H $\alpha$ 122-210, all including the binding site, but it did not bind to H $\alpha$ 1-121, H $\alpha$ 1-121+p3A nor to the GST protein (Fig. 3C). As shown, both mAb 198 and mAb 5.5 bound better to the variants containing the sequence encoded by the p3A exon.

The binding of mAb 198 to the polypeptides of the invention was also determined in ELISA carried out as described in Materials and Methods section (ii), and the results are shown in Fig. 4. In this assay, as in the Western blot, mAb 198 bound better to the polypeptides H $\alpha$ 1-210+p3A and H $\alpha$ 1-121+p3A (filled symbols). Therefore, these longer variants were used in further studies. Three other anti-MIR mAbs (mAb 195, mAb 202 and mAb 35) bound to a lesser extent than mAb 198 to all tested polypeptides (not shown).

Fig. 5 illustrates the binding of various mAbs to H $\alpha$ 1-210+p3A: Mab 198 (filled squares) showed a very strong binding; MAb 35, which is directed against the MIR and is known to depend on the native conformation of AChR, showed very low binding to the tested polypeptides of the invention (open circles). MAb 5.5 which also depends on the native conformation of AChR, bound well to the tested polypeptides in Western blots (Fig. 3C), but to a much lesser extent than mAb 198 in ELISA (open triangles). This poor binding of mAbs 35 and 5.5 may indicate that when bound to ELISA plates only a small fraction of the recombinant polypeptide is properly folded.

Based on the results of the binding experiments in ELISA, the next step was to test whether the polypeptides of the invention bind to the mAbs also in solution. For that, the ability of the various recombinant polypeptides to inhibit the binding of mAb 198 to *Torpedo* AChR was tested in ELISA. As shown in Fig 6, H $\alpha$ 1-210+p3A (filled squares) and H $\alpha$ 1-121+p3A (filled circles) inhibited this binding, with IC<sub>50</sub> values of  $1.8 \times 10^{-7}$ M and  $1 \times 10^{-7}$ M, respectively, whereas the GST protein (filled triangles) did not, indicating that the solubilized recombinant fused polypeptides may indeed bind to mAb 198 also in solution. As shown above (Fig. 3B and Fig. 4), the variants containing the additional 25 amino acid residues encoded by the p3A exon were more potent in inhibiting mAb 198 binding to AChR than their counterparts lacking this 25-mer.

#### **Example 4: Effect of the polypeptides on antigenic modulation of AChR in TE671 cells**

Muscle AChR loss in myasthenia is caused by accelerated degradation of the receptor, brought about by anti-AChR antibodies, a great portion of which are directed to the MIR. This activity of the antibodies can be demonstrated *in vitro* in cell cultures such as the human cell line TE671. This human medulloblastoma-derived cell line expresses a functional acetylcholine receptor which binds  $\alpha$ -bungarotoxin ( $\alpha$ -BTX) and has the  $\alpha$ -subunit of the muscle-type AChR. The ability of the recombinant polypeptides H $\alpha$ 1-210 and H $\alpha$ 1-121 to protect the AChR on TE671 cells against accelerated degradation of AChR induced by specific anti-AChR  $\alpha$ -subunit mAbs, was examined as follows: Anti-MIR mAbs were preincubated for one hour at 37°C with several concentrations of the recombinant polypeptide and then added to the cells. As a control, the mAbs were preincubated with GST or with the H $\alpha$ 122-210 polypeptide that does not include the MIR. The inhibition effect of H $\alpha$ 1-121 on AChR degradation induced by mAb 198 measured as residual  $\alpha$ -BTX binding sites, is illustrated in Fig. 7. MAb 198 causes a reduction

of 41% in residual AChR following 3 h incubation with the cells (Fig. 7, lane b). Preincubation with increasing concentrations of H $\alpha$ 1-121 had a dose dependent protection effect against the degradation induced by mAb 198 (Fig. 7, c-g, hatched columns). At a concentration of 100 $\mu$ g/ml of H $\alpha$ 1-121 the TE671 cells were completely protected against the accelerated AChR degradation by mAb 198. Preincubation of mAb 198 with H $\alpha$ 122-210, which does not contain the MIR, did not affect the antigenic modulation induced by mAb 198 and did not block AChR degradation (Fig. 7, c-g, dark columns). H $\alpha$ 1-210, corresponding to the entire extracellular  $\alpha$ -subunit domain, had the same effect as the shorter fragment H $\alpha$ 1-121 (data not shown).

Results of a comparable experiment carried out with other anti-AChR mAbs are shown in Fig. 8. The polypeptide H $\alpha$ 1-121 had a similar protection effect against AChR degradation induced by two other anti-MIR mAbs, mAb 195 and mAb 202, but had a much smaller effect on mAb 35- induced AChR degradation, possibly because of the weak binding of this antibody to H $\alpha$ 1-121 in solution (see Fig. 5).

#### **Example 5: Modulation by the polypeptides of EAMG passively transferred by mAb 198**

The effect of the polypeptides of the invention was also examined in vivo in a well-established animal model disease for myasthenia gravis, designated experimental autoimmune myasthenia gravis (EAMG) [Lindstrom et al., 1976; Lindstrom et al., 1976a]. In animals such as rabbits, mice, guinea-pigs, monkeys and rats EAMG can be either passively transferred by anti-AChR antibodies, or actively induced by AChR. In both cases, the treated animals show chronic symptoms of the MG disease, i.e. show general weakness, have a hunched posture, develop a flaccid paralysis of the hind limbs, have difficulties in breathing, in swallowing and in reaching food and water supplied to them, all of which result in weight loss. The animals die from respiratory insufficiency, malnutrition and dehydration. In rats, two distinct episodes of weakness occur, especially after immunization with Torpedo AChR in combination with *Mycobacterium tuberculosis* (killed) H37 Ra, with an acute phase starting 8-10 days after immunization and a chronic phase starting 3-5 weeks later.

Table 1: Recombinant fragments modulate experimental myasthenia passively transferred by a monoclonal anti AChR antibody.

Treatment	Anti-AChR mAb 198	Myasthenic symptoms	AChR content* fmoles/mg prot.	% of control
-	-	-	39.9±6.3	100
-	+	+	19.2±3.5	48
H $\alpha$ 1-121	+	-	38.8±6.9	97
H $\alpha$ 122-210	+	+	24.5±2.4	61
GST	+	+	19.2±4.5	48
BSA	+	+	21.4±2.4	53

\*Muscle AChR content was determined by  $\alpha$ -bungarotoxin binding to AChR present in Triton X-100 extracts from rat leg muscles, 48 h after Ig administration.

The values ( mean±SEM) are averages derived from at least three different animals.

EAMG was passively transferred in rats by mAb 198. The disease was induced within 24-48 hrs following administration of the antibody [Asher et al., 1993]. Muscle AChR content was determined by  $\alpha$ -bungarotoxin binding to AChR present in Triton X-100 extracts from rat leg muscles, 48 h after the mAb administration. As previously reported, the myasthenic symptoms were accompanied by a marked reduction in the muscle AChR content (48% of normal control; Table 1). In order to examine the effect of the polypeptides of the invention on the disease symptoms, mAb 198 was preincubated with a 30 fold molar excess of recombinant polypeptides of the invention, or with either GST or BSA as controls, prior to its injection into rats.

As shown in Table 1, the muscle AChR content in the EAMG-induced rats was reduced to 48% of AChR content of control untreated rats. The recombinant polypeptides of the invention were able to modulate in vivo muscle AChR loss and to decrease significantly clinical symptoms of EAMG. It was shown that preincubation of mAb 198 with H $\alpha$ 1-121+p3A prior to its injection into rats, prevented the appearance of myasthenic symptoms. The protected rats had a normal muscle AChR content (97% of control). Similar results were obtained with the H $\alpha$ 1-210+p3A polypeptide (data not shown). On the other hand, preincubation with either H $\alpha$ 122-210+p3A or with GST or BSA did not affect the muscle AChR content significantly (61, 48 and 53% of control, respectively) and did not prevent myasthenic symptoms. Administration of  $\alpha$ 1-121+p3A and H $\alpha$ 122-210+p3A alone did not induce any myasthenic symptoms in rats.

Interestingly, similar protection effect by H $\alpha$ 1-121+p3A and H $\alpha$ 1-210+p3A was demonstrated when the recombinant polypeptide was injected together with mAb 198 without preincubation, or even two hours after the administration of mAb 198 (data not shown).

**Example 6: Effects of nasal administration of the polypeptides of the invention on actively induced EAMG in rats**

H $\alpha$ 1-210+p3A, H $\alpha$ 1-121+p3A and H $\alpha$ 122-210 fused with glutathione S-transferase (GST) were expressed and solubilized as described in Example 2. Nasal tolerance was induced in rats by administration of a daily dose of 2.5 $\mu$ g of each of said fused polypeptides in 30  $\mu$ l PBS into each rat nostril, over a period of ten consecutive days. Three days later the rats were immunized with Torpedo AChR (40  $\mu$ g/rat) injected into the footpads, in Complete Freund's Adjuvant supplemented with 1 mg of M. tuberculosis H37RA (DIFCO). Control rats received GST instead of the recombinant polypeptide. Clinical symptoms of EAMG disease, as well as

body weight, were monitored weekly. The results of the experiment are summarized in Table 2, showing that all three tested polypeptides had a protective effect in the rats. Two weeks after the immunization with AChR, nine out of ten control rats developed clear symptoms of EAMG, whereas only four out of 36 rats, that received the different polypeptides, developed clinical symptoms of the disease. There were no significant differences between the groups that were pretreated with H $\alpha$ 1-210+p3A, H $\alpha$ 1-121+p3A or H $\alpha$ 122-210. A similar situation was observed five weeks after the immunization with AChR; 10/10 sick or dead rats in the control group and only 7/36 sick rats in the nasally pretreated rats.

The pretreated rats did not develop measurable serum antibodies to the recombinant polypeptides, to AChR or to GST, prior to the immunization with AChR. Following the immunization with AChR, all rats developed comparable antibody titers towards Torpedo AChR.

**Table 2: Clinical course of EAMG in rats nasally tolerized with Human AChR recombinant fragments**

Pretreatment	<u>EAMG</u>			
	<u>2wk*</u> (No./Total)		<u>5wk*</u> (No./Total)	
	<u>protected</u>	<u>sick</u>	<u>protected</u>	<u>sick</u>
H $\alpha$ 1-210 +p3A	10/12	2/12	10/12	2/12
H $\alpha$ 1-121 +p3A	11/12	1/12	9/12	3/12
H $\alpha$ 122-210	11/12	1/12	10/12	2/12
GST	1/10	9/10	0/10	10/10

\* time after AChR injection.

Nasal administration to rats of the polypeptides of the invention affected also the in vitro T-lymphocyte proliferative response to AChR. Rats were pretreated with either GST (control), H $\alpha$ 122-210, H $\alpha$ 1-121+p3A or H $\alpha$ 1-210+p3A, and immunized 3 days later with Torpedo AChR, as described above. Three weeks after immunization, the rats were sacrificed and their lymph nodes were restimulated in vitro by 5.0, 2.5 and 0.25  $\mu$ g/ $\mu$ l AChR. Results are depicted in Fig. 9, where the proliferative response is presented as [ $^3$ H]-thymidine cpm incorporated into cells after subtraction of cpm in the presence of medium alone.

As shown in Fig. 9, rats pretreated intranasally with either H $\alpha$ 1-210 or H $\alpha$ 1-121 had almost no proliferative response to Torpedo AChR. T-lymphocytes from rats pretreated with H $\alpha$ 122-210 responded to a lower extent than T-lymphocytes from control rats when restimulated with 0.25 or 2.5  $\mu$ g/ml AChR, and did not respond at all when exposed to 5 $\mu$ g/ml AChR.

These protection effects, shown both in vivo and in vitro, suggest that the polypeptides of the invention affect the autoimmune response to AChR in a manner that may be employed for immunotherapy of myasthenia gravis. Thus, the nasal route of administration could provide a convenient therapeutic modality in humans.

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## CLAIMS

1. A polypeptide capable of modulating the autoimmune response of an individual to acetylcholine receptor, said polypeptide being selected from the group consisting of:

(i) a polypeptide corresponding to amino acid residues 1-210 of the human acetylcholine receptor (hAChR)  $\alpha$ -subunit sequence depicted in Fig.1 ( $H\alpha 1$ -210), in which is inserted, between amino acid residues 58 and 59, a sequence of 25 amino acid residues encoded by the p3A exon of the hAChR  $\alpha$ -subunit gene, depicted in Fig. 2 ( $H\alpha 1$ -210+p3A);

(ii) a polypeptide corresponding to amino acid residues 1-121 of the hAChR  $\alpha$ -subunit sequence depicted in Fig.1 ( $H\alpha 1$ -121);

(iii) a polypeptide corresponding to amino acid residues 1-121 of the hAChR  $\alpha$ -subunit sequence depicted in Fig.1, in which is inserted, between amino acid residues 58 and 59, a sequence of 25 amino acid residues encoded by the p3A exon of the hAChR  $\alpha$  subunit gene, depicted in Fig. 2 ( $H\alpha 1$ -121+p3A);

(iv) a polypeptide corresponding to amino acid residues 122-210 of the hAChR  $\alpha$ -subunit sequence depicted in Fig.1 ( $H\alpha 122$ -210);

(v) a polypeptide as in (i) to (iv) or the polypeptide  $H\alpha 1$ -210 in which one or more amino acid residues have been added, deleted or substituted by other amino acid residues in a manner that the resulting polypeptide is capable of modulating the autoimmune response to acetylcholine receptor;

(vi) a fragment of a polypeptide as in (i) to (v), which fragment is capable of modulating the autoimmune response to acetylcholine receptor;

(vii) a polypeptide comprising two or more fragments as in (vi) fused together with or without a spacer;

(viii) a polypeptide or a fragment as defined in (i)-(vii) or the polypeptide  $H\alpha 1$ -210 fused to an additional polypeptide at its N- and/or C-terminal; and

(ix) soluble forms, chemical derivatives and salts of a polypeptide or a fragment as defined in (i)-(viii).

2. A polypeptide according to claim 1 corresponding to amino acid residues 1-210 of the hAChR  $\alpha$ -subunit sequence depicted in Fig.1, in which is inserted, between amino acid residues 58 and 59, a sequence of 25 amino acid residues encoded by the p3A exon of the hAChR  $\alpha$ -subunit gene depicted in Fig. 2.

3. A polypeptide according to claim 1, corresponding to amino acid residues 1-121 of the hAChR  $\alpha$ -subunit sequence depicted in Fig.1.

4. A polypeptide according to claim 1, corresponding to amino acid residues 1-121 of the hAChR  $\alpha$ -subunit sequence depicted in Fig.1, in which is inserted, between amino acid residues 58 and 59, a sequence of 25 amino acid residues encoded by the p3A exon of the hAChR  $\alpha$ -subunit gene depicted in Fig.2.

5. A polypeptide according to claim 1, corresponding to amino acid residues 122-210 of the hAChR  $\alpha$ -subunit sequence depicted in Fig.1.

6. A polypeptide or a fragment according to claim 1(viii) wherein the additional polypeptide is glutathione S-transferase (GST) fused at the N-terminal of a polypeptide according to claim 1 (i) - (vii).

7. A DNA molecule coding for a polypeptide or a fragment according to claim 1.

8. A DNA molecule according to claim 7, being selected from the group consisting of:

(i) a DNA molecule comprising the sequence of nucleotides 1 to 630, depicted in Fig.1, in which the sequence of the p3A exon of the hAChR  $\alpha$ -subunit gene, depicted in Fig. 2, is inserted between nucleotides 174 and 175;

(ii) a DNA molecule comprising the sequence of nucleotides 1 to 363 depicted in Fig.1;

(iii) a DNA molecule comprising the sequence of nucleotides 1 to 363 depicted in Fig.1, in which the sequence of the p3A exon of the hAChR  $\alpha$ -subunit gene, depicted in Fig. 2, is inserted between nucleotides 174 and 175;

(iv) a DNA molecule comprising the sequence of nucleotides 364 to 630 depicted in Fig.1;

(v) DNA molecules which are degenerate, as a result of the genetic code, to the DNA sequences of (i) to (iv) and which code for a polypeptide coded for by any one of the DNA sequences of (i) to (iv);

(vi) a DNA molecule having a coding nucleotide sequence which is at least 70% homologous to any one of the DNA sequences of (i) to (v) or to the DNA sequence coding for H $\alpha$ 1-210;

(vii) a DNA molecule as in (i) to (iv) or the DNA molecule coding for H $\alpha$ 1-210 in which one or more codons has been added, replaced or deleted in a manner that the polypeptide coded for by said sequence is capable of modulating the autoimmune response to acetylcholine receptor;

(viii) a fragment of a DNA molecule as in (i)-(vii) which codes for a polypeptide capable of modulating the autoimmune response to acetylcholine receptor;

(ix) a DNA molecule comprising two or more fragments of (viii) fused together with or without a spacer, and which codes for a polypeptide capable of modulating the autoimmune response to acetylcholine receptor; and

(x) a DNA molecule comprising a nucleic acid sequence as defined in (i)-(ix) or the DNA sequence coding for H $\alpha$ 1-210 fused to additional coding DNA sequences at its 3' and/or 5' end.

9. A DNA molecule according to claim 8 which comprises the sequence of nucleotides 1 to 630, depicted in Fig.1, in which the sequence of the p3A exon of the hAChR  $\alpha$ -subunit gene, depicted in Fig.2, is inserted between nucleotides 174 and 175, said DNA molecule coding for the polypeptide according to claim 2

10. A DNA molecule according to claim 8 which comprises the sequence of nucleotides 1 to 363 of the DNA sequence depicted in Fig.1, said DNA molecule coding for the polypeptide according to claim 3.

11. A DNA molecule according to claim 8 which comprises the sequence of nucleotides 1 to 363 of the DNA sequence depicted in Fig.1, in which a nucleic acid sequence of the p3A exon of the hAChR  $\alpha$ -subunit gene, depicted in Fig. 2, is inserted between nucleotides 174 and 175, said DNA molecule coding for the polypeptide according to claim 4.

12. A DNA molecule according to claim 8 which comprises the sequence of nucleotides 364 to 630 of the DNA sequence depicted in Fig.1, said DNA molecule coding for the polypeptide according to claim 5.

13. A DNA molecule according to claim 1 (x) wherein said additional nucleic acid sequence codes for glutathione S-transferase (GST) and is fused at the 5' end, said fused DNA molecule coding for a polypeptide according to claim 6.

14. A replicable expression vehicle comprising a DNA molecule according to any one of claims 7-13.

15. A prokaryotic or eukaryotic host cell transformed by a replicable expression vehicle of claim 14.

16. A process for preparing a polypeptide of any one of claims 1-6, comprising:

- (i) culturing a host cell of claim 15 under conditions promoting expression, and
- (ii) isolating the expressed polypeptide.

17. A process according to claim 16, wherein the expressed polypeptide is a fused polypeptide.

18. A pharmaceutical composition comprising a pharmaceutically acceptable carrier and a polypeptide selected from a polypeptide according to any one of claims 1 to 6 and a polypeptide comprising the amino acid residues 1-210 of the hAChR  $\alpha$ -subunit depicted in Fig.1, soluble forms, salts and chemical derivatives thereof.

19. A pharmaceutical composition according to claim 18 for alleviation and/or treatment of myasthenia gravis.

20. A pharmaceutical composition according to claim 19 for nasal or oral administration.

21. A method for alleviation and/or treatment of myasthenia gravis which comprises administering to an individual in need thereof, an effective amount of a polypeptide according to any one of claims 1 to 6 or a polypeptide comprising the amino acid residues 1-210 of the hAChR  $\alpha$ -subunit depicted in Fig.1, soluble forms, salts and chemical derivatives thereof.

22. A pharmaceutical composition according to claim 18 for diagnosis of myasthenia gravis.

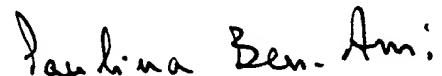
23. A method for diagnosis of myasthenia gravis comprising:

(i) incubating one or more polypeptides selected from a polypeptide according to any one of claims 1 to 6 and a polypeptide comprising the amino acid residues 1-210 of the hAChR  $\alpha$ -subunit depicted in Fig.1, soluble forms, salts and chemical derivatives thereof, with a serum aliquote from an individual; and

(ii) determining the amount of the anti-AChR antibodies in the serum bound to said polypeptide of (i);

whereby detection of anti-AChR titers indicates the presence of myasthenia gravis.

For the Applicants:



Paulina Ben-Ami

Patent Attorney

Fig 1

1 TCC GAA CAT GAG ACC CGT CTG GTG GCA AAG CTA TTT AAA GAC TAC 45  
1 Ser Glu His Glu Thr Arg Leu Val Ala Lys Leu Phe Lys Asp Tyr 15

46 AGC AGC GTG GTG CGG CCA GTG GAA GAC CAC CGC CAG GTC GTG GAG 90  
16 Ser Ser Val Val Arg Pro Val Glu Asp His Arg Gln Val Val Glu 30

91 GTC ACC GTG GGC CTG CAG CTG ATA CAG CTC ATC AAT GTG GAT GAA 135  
31 Val Thr Val Gly Leu Gln Leu Ile Gln Leu Ile Asn Val Asp Glu 45

136 GTA AAT CAG ATC GTG ACA ACC AAT GTG CGT CTG AAA CAG CAA TGG 180  
46 Val Asn Gln Ile Val Thr Thr Asn Val Arg Leu Lys Gln Gln Trp 60

181 GTG GAT TAC AAC CTA AAA TGG AAT CCA GAT GAC TAT GGC GGT GTG 225  
61 Val Asp Tyr Asn Leu Lys Trp Asn Pro Asp Asp Tyr Gly Gly Val 75

226 AAA AAA ATT CAC ATT CCT TCA GAA AAG ATC TGG CGC CCA GAC CTT 270  
76 Lys Lys Ile His Ile Pro Ser Glu Lys Ile Trp Arg Pro Asp Leu 90

271 GTT CTC TAT AAC GAT GCA GAT GGT GAC TTT GCT ATT GTC AAG TTC 315  
91 Val Leu Tyr Asn Asp Ala Asp Gly Asp Phe Ala Ile Val Lys Phe 105

316 ACC AAA GTG CTC CTG CAG TAC ACT GGC CAC ATC ACG TGG ACA CCT 360  
106 Thr Lys Val Leu Leu Gln Tyr Thr Gly His Ile Thr Trp Thr Pro 120

361 CCA GCC ATC TTT AAA AGC TAC TGT GAG ATC ATC GTC ACC CAC TTT 405  
121 Pro Ala Ile Phe Lys Ser Tyr Cys Glu Ile Ile Val Thr His Phe 135

406 CCC TTT GAT GAA CAG AAC TGC AGC ATG AAG CTG GGC ACC TGG ACC 450  
136 Pro Phe Asp Glu Gln Asn Cys Ser Met Lys Leu Gly Thr Trp Thr 150

451 TAC GAC GGC TCT GTC GTG GCC ATC AAC CCG GAA AGC GAC CAG CCA 495  
151 Tyr Asp Gly Ser Val Val Ala Ile Asn Pro Glu Ser Asp Gln Pro 165

496 GAC CTG AGC AAC TTC ATG GAG AGC GGG GAG TGG GTG ATC AAG GAG 540  
166 Asp Leu Ser Asn Phe Met Glu Ser Gly Glu Trp Val Ile Lys Glu 180

541 TCC CGG GGC TGG AAG CAC TCC GTG ACC TAT TCC TGC TGC CCC GAC 585  
181 Ser Arg Gly Trp Lys His Ser Val Thr Tyr Ser Cys Cys Pro Asp 195

586 ACC CCC TAC CTG GAC ATC ACC TAC CAC TTC GTC ATG CAG CGC CTG 630  
196 Thr Pro Tyr Leu Asp Ile Thr Tyr His Phe Val Met Gln Arg Leu 210

Fig 2

GGT GAC ATG GTA GAT CTG CCA CGC CCC AGC TGC GTG ACT TTG GGA  
Gly Asp Met Val Asp Leu Pro Arg Pro Ser Cys Val Thr Leu Gly

GTT CCT TTG TTT TCT CAT CTG CAG GAT GAG  
Val Pro Leu Phe Ser His Leu Gln Asp Glu

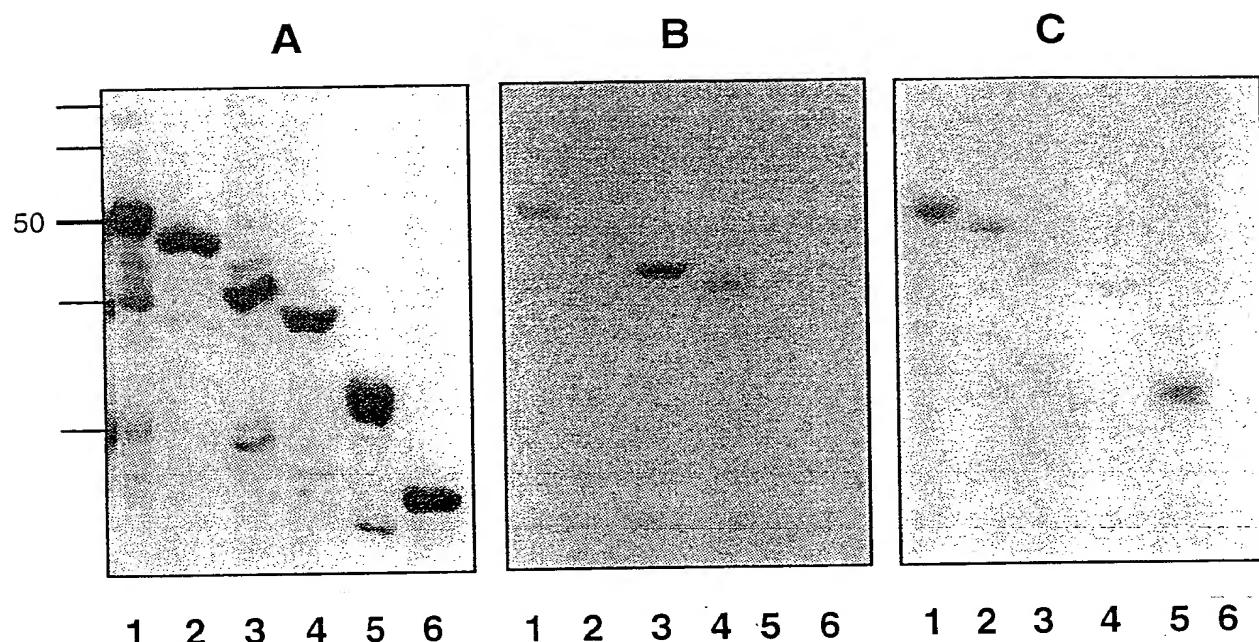


Fig 3.

Fig 4

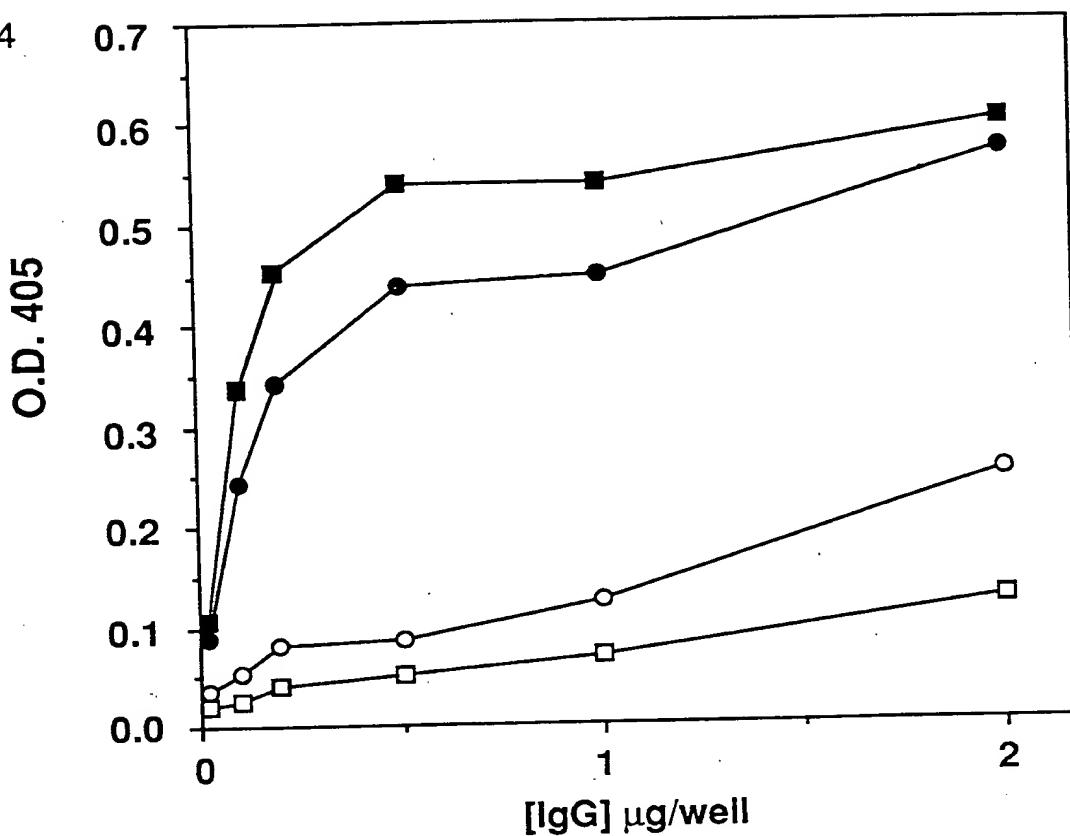
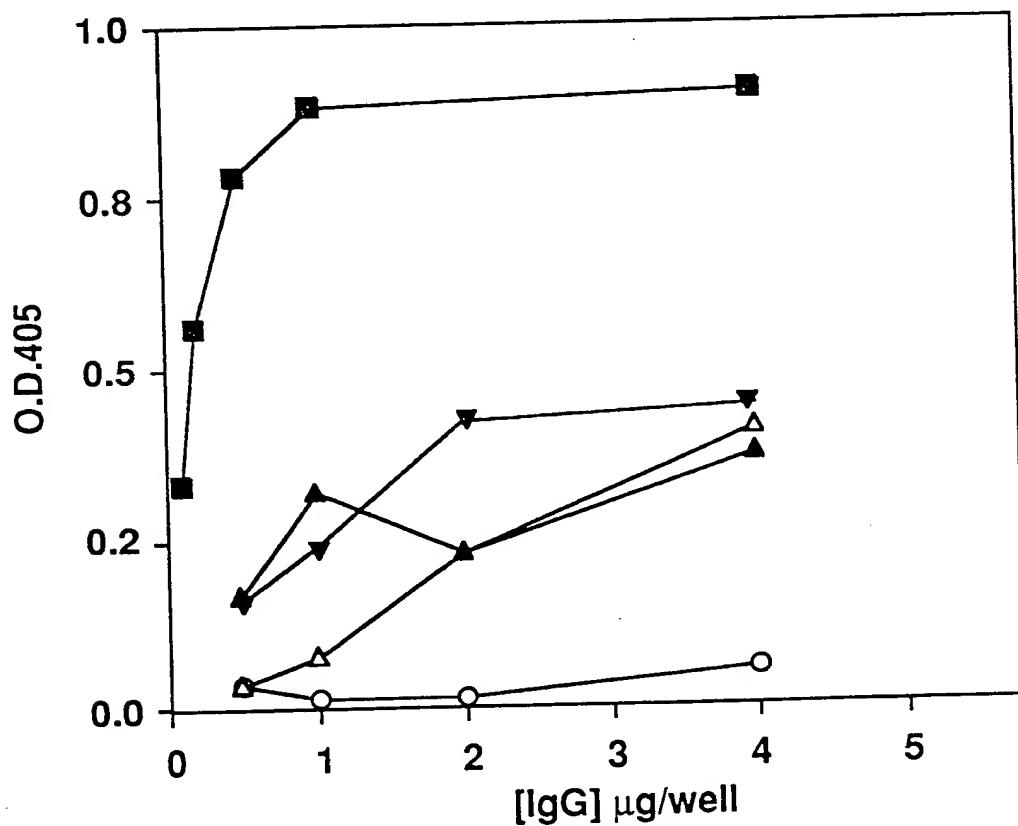


Fig 5



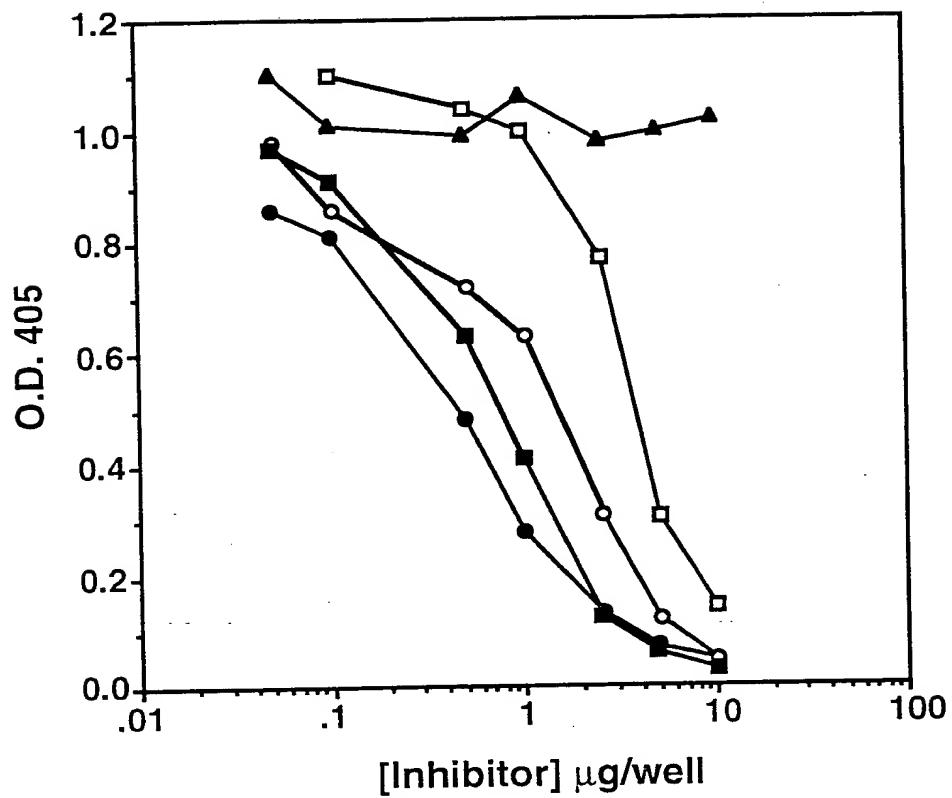


Fig 6

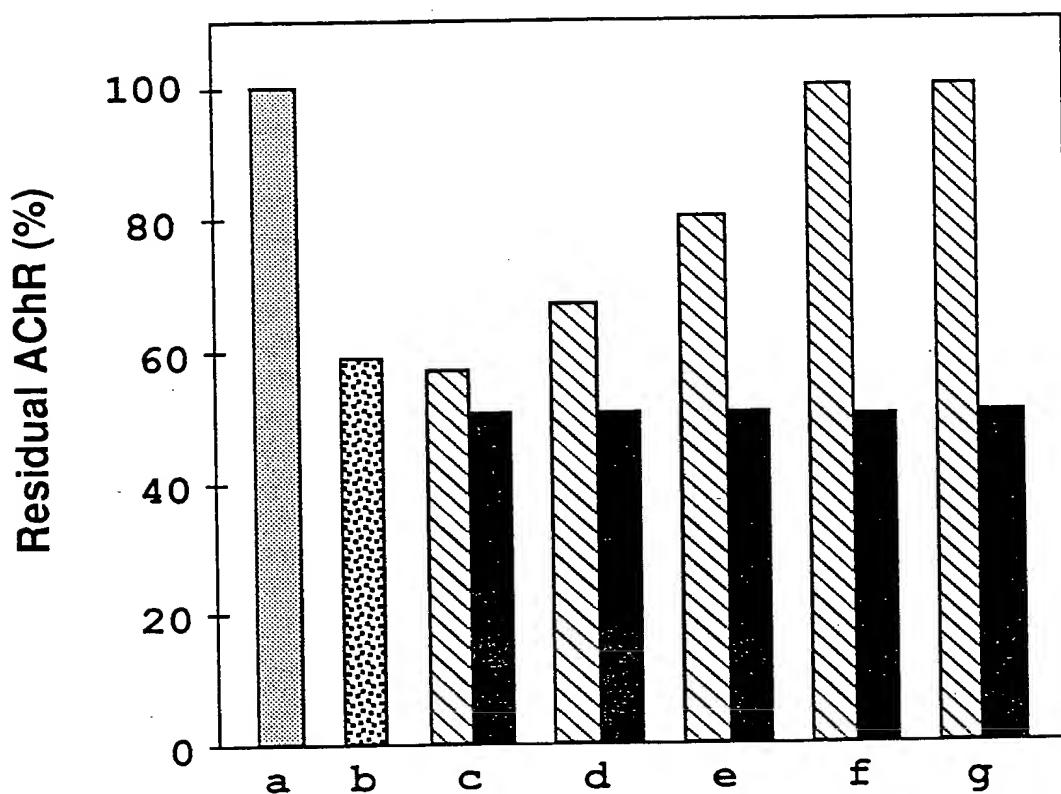


Fig7

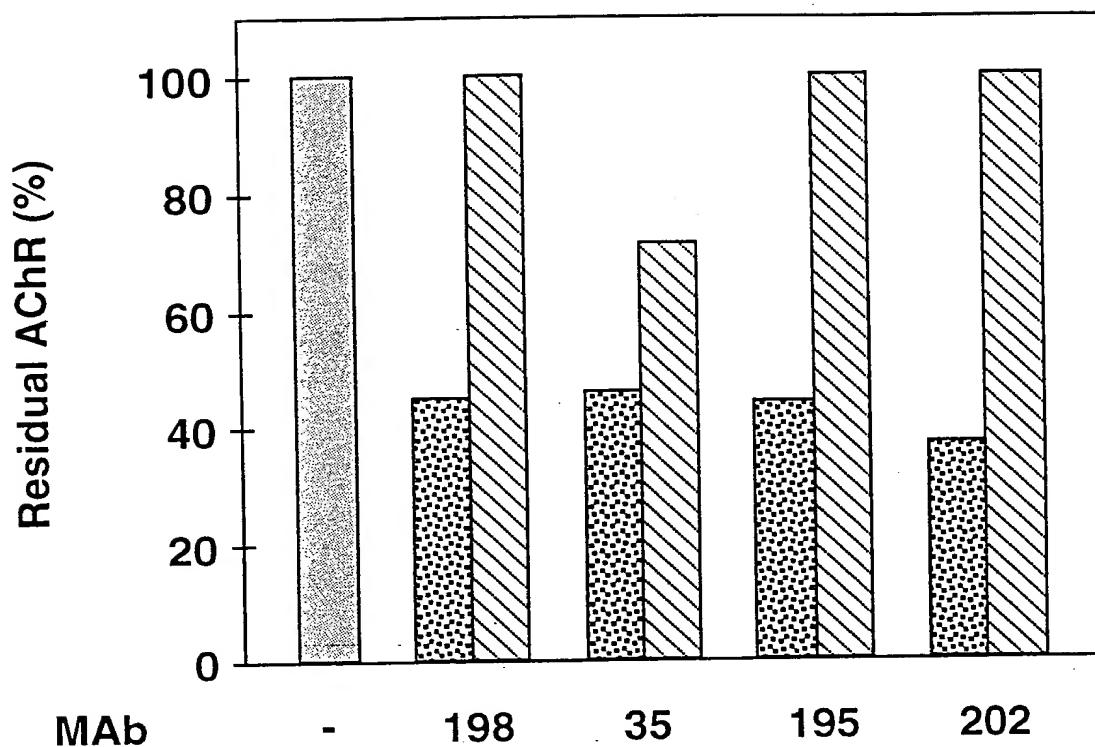


Fig 8

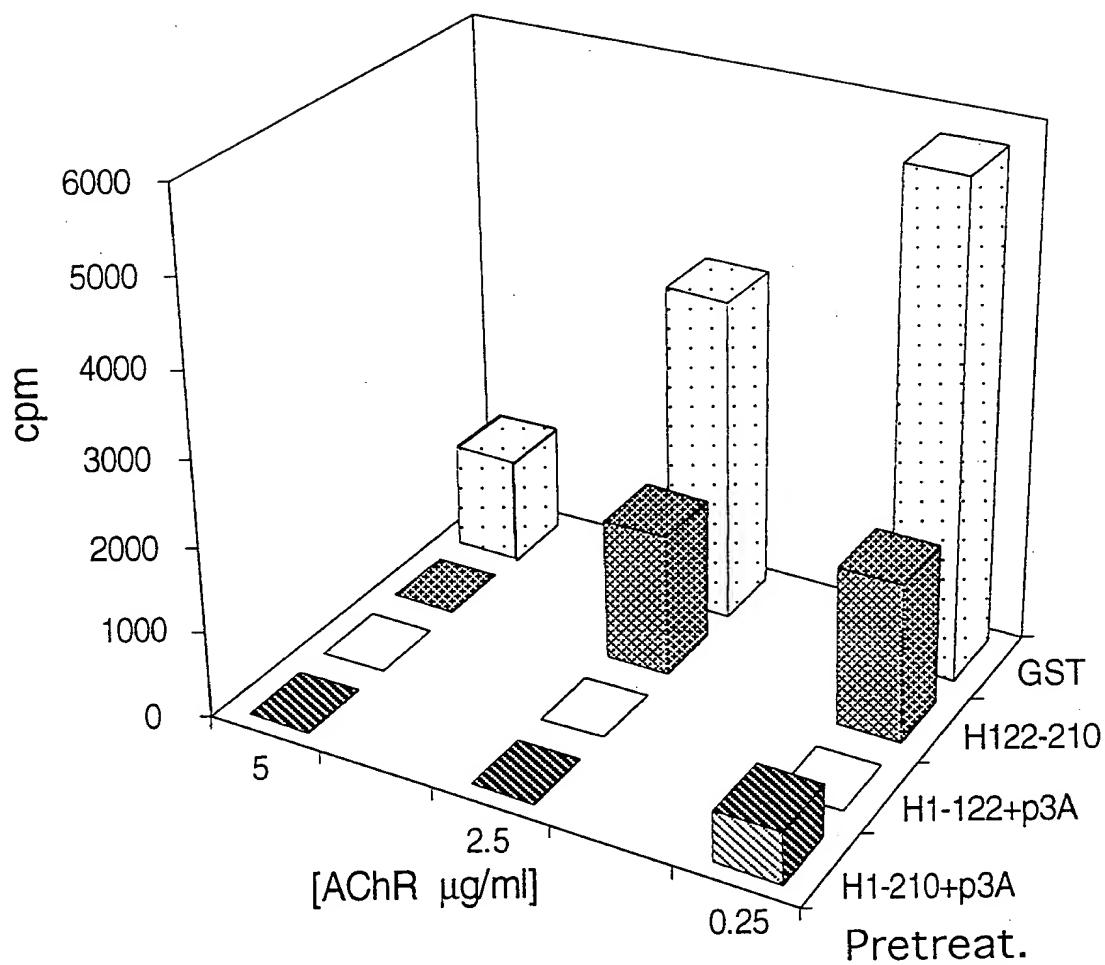


Fig 9